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14. ABSTRACT

A critical problem in prostate cancer is our inability to reliably distinguish indolent from aggressive disease. Recent evidence has implicated a class of genes, termed Cancer Testis Antigens (CTA), in cancer progression. In preliminary studies, by crossing the CTA bank with a prostate cancer metastasis gene signature we attained from an orthotopical injection xenograft model, we postulated CTA SPANXB2 as a cancer metastasis related CTA. We observed that SPANXB2 is up-regulated in metastatic prostate cancer xenograft models and is induced upon exposure to stroma and stromal factors (i.e., TGF-β). We hypothesize that SPANX-B2 may be the key regulator of prostate cancer aggressive cell behavior and metastasis. In this report, for the first time, we illustrate that regulatory role of SPANXB2 in PC3 cells by using shRNA knockdown technique. Knockdown of SPANXB2 in PC3 cells significantly reduces the cell proliferation, migration, and invasion ability compared with the wild type PC3 cells. Additionally, co-culture of these knockdown cells with stromas partially rescues the phenotype. We also confirm that stromal cells promote cell aggressiveness in prostate caner cells and detect the TGF-β2 secretion is correlated to elevated SPANXB2 level in our epithelial-stromal model. The attained data provide solid basis for the second stage in vivo studies.

15. SUBJECT TERMS
SPANXB2, Prostate Cancer, Stromas, Metastasis

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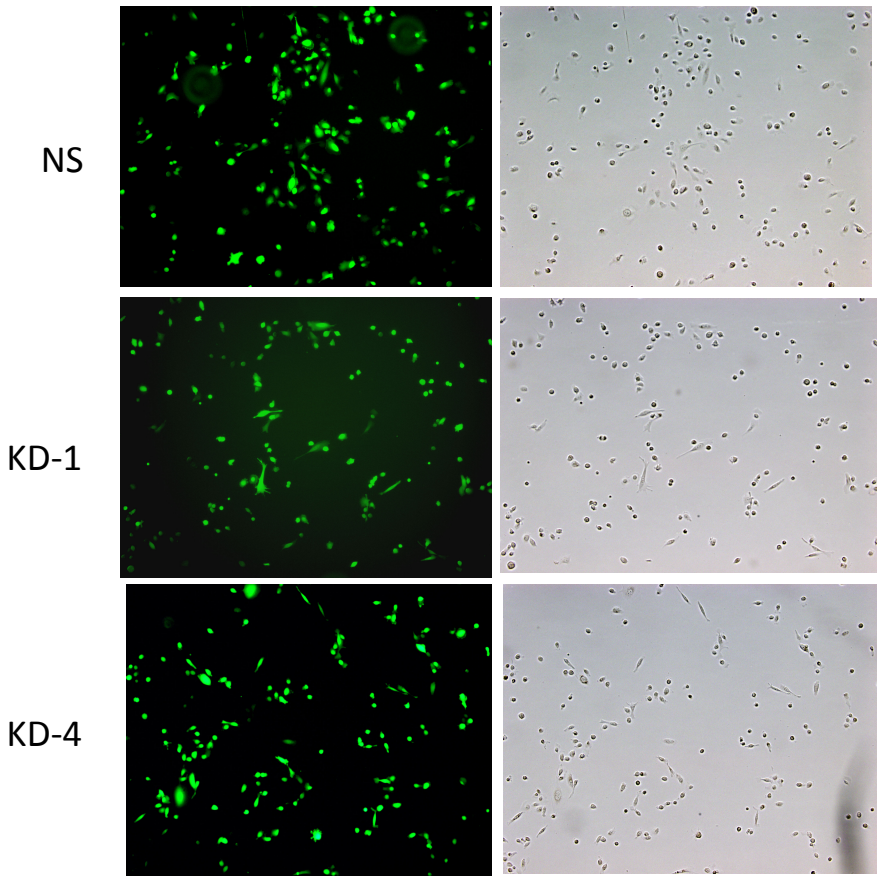
Introduction

A critical problem in prostate cancer is our inability to reliably distinguish indolent from aggressive disease. Cancer metastasis gene, but not tumor initiating gene, might be the potential target to solve this problem. Recent evidence has implicated a class of genes, termed Cancer Testis Antigens (CTA), in cancer progression. In preliminary studies, by crossing the CTA bank with a prostate cancer metastasis gene signature we attained from an orthotopical injection xenograft model, we postulated CTA SPANXB2 as a cancer metastasis related CTA. We observed that SPANXB2 is up-regulated in metastatic prostate cancer xenograft models and is induced upon exposure to stroma and stromal factors (i.e., TGF-β). We hypothesize that SPANX-B2 may be the key regulator of prostate cancer aggressive cell behavior and metastasis. We also hypothesize that prostate stroma promotes SPANX-B2 expression in prostate cancer cells *in vivo* and *in vitro,* and most likely, this process is modulated by TGFβ. Finally we seek to the link between SPANX-B2 expression and clinical marker including recurrence (PSA), lymph node metastasis, prostate cancer specific death and reactive stroma grade. In order to accomplish these aims, we plan to complete the determining the association of SPANX-B2 and stroma with prostate cancer aggressiveness *in vitro* in the first year's fellowship and finish **ii**) Determine the association of SPANX-B2 and stroma with prostate cancer metastasis *in vivo* and **iii**) Test the association of SPANX-B2 expression with biochemical recurrence (PSA), lymph node metastasis, prostate cancer specific death and reactive stroma grade (RSG) at the second year.

Body

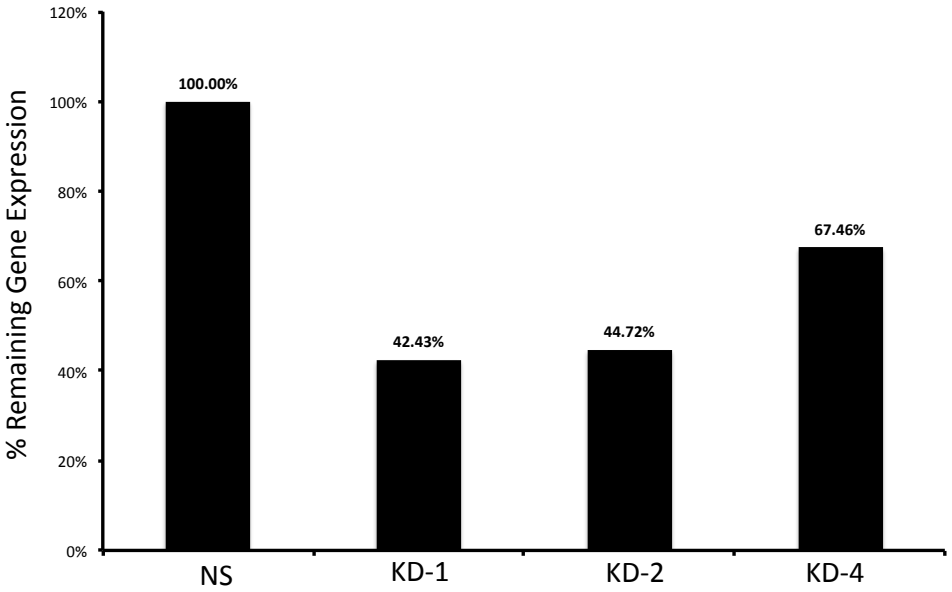
Figure 1



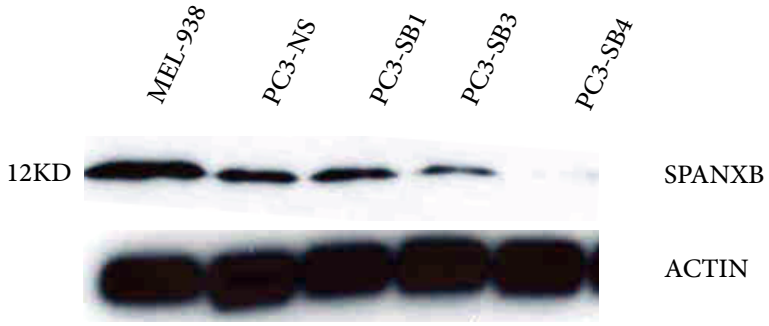
Summary: Figure 1 is linked to the **Task 1a : a) in my research plan,** images of SPANXB2 knockdown cell lines are indicted here (with a GFP and phase image for each cell lines). GIPZ vector are purchased form Openbiosystems, NS is the non-silence backbone control, KD-1 and KD-4 are two different knockdown stable line. These results indicated that the establishment of PC3-SPANXB2 Knock down stable line.

Figure 2

A



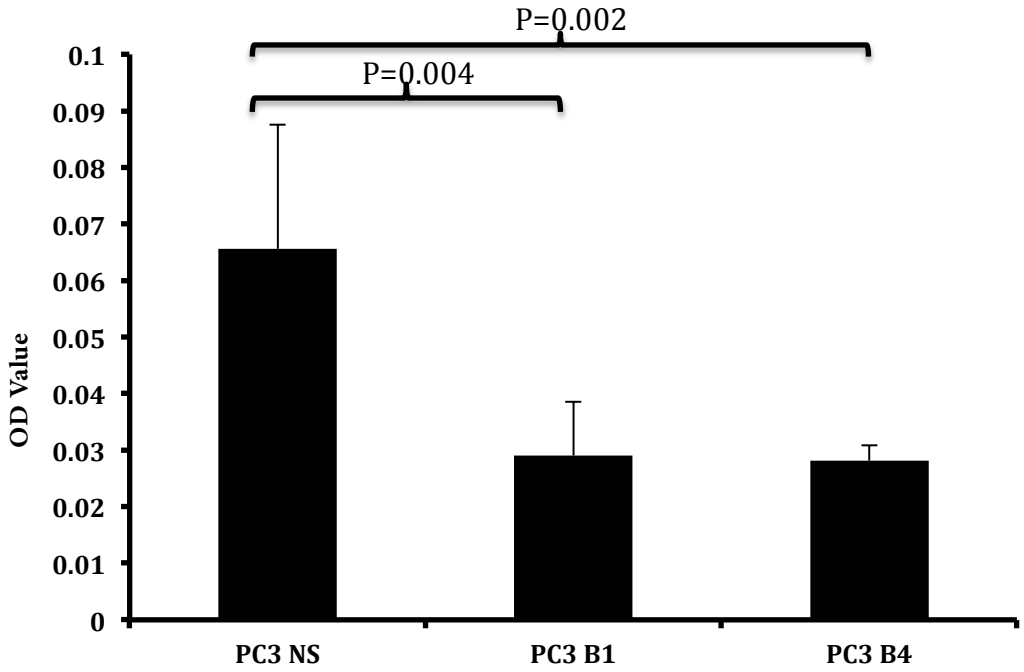
B



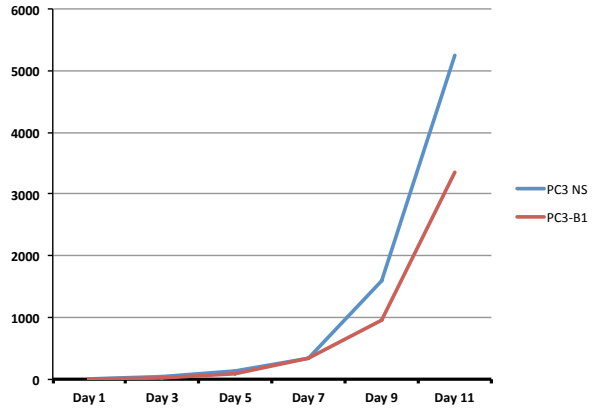
Summary: Figure 2. A, B are linked to the **Task 1a, d:** (A): SPANXB2 mRNA level were detected in PC3-NS, PC3-KD-1,2,4 lines respectively by using Real Time qPCR. SPANXB2 mRNA were significantly knockdown about 58%, 57% and 33% in PC3-KD-1,2, and 4 respectively. (B): Western Blotting is performed to confirm the KD effect of SPANXB. Anti-SPANXB antibody is a gift from Dr. Kouprina from NIH, we also followed up the same protocol from their papers (1, 2). Mel-938 is a melanoma line and used as a positive control. <u>These experiment demonstrated that the successfully SPANXB2 knockdown effect on both mRNA level and protein level in PC3 cells.</u>

Figure 3

A

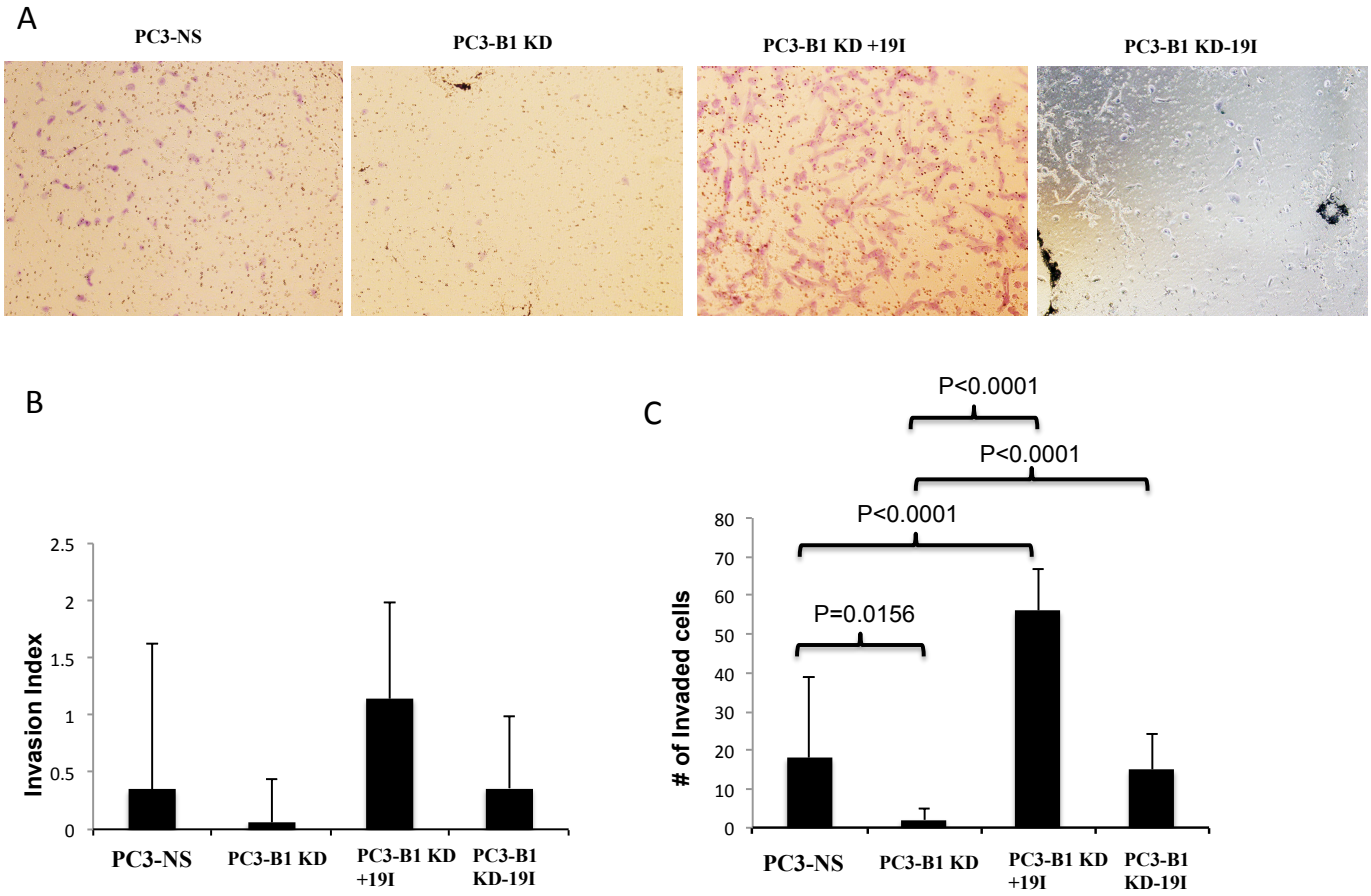


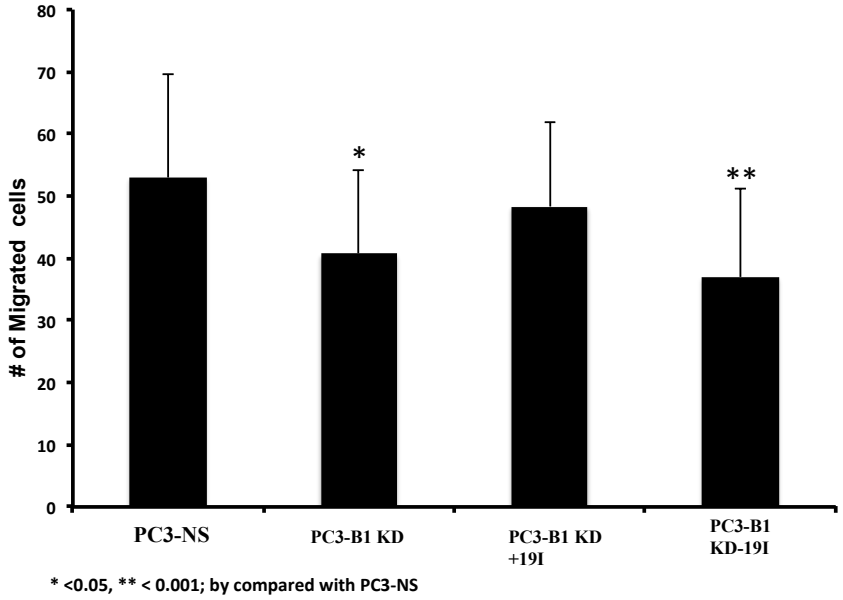
B



Summary: Figure 3. A is linked to the **Task 1a, e:** The proliferation potential of SPANXB2 knockdown stable lines were measured by MTT assay. PC3-B1, (PC3-B1=KD-1, PC3-B4= KD-4), as well as PC3-B4, indicated reduced proliferation potential by comparing with PC3-NS. B) Cell population doubling of PC3 NS and PC3-SB4 were performed. A similar result was attained to indicate that PC3-SB1 has a reduced proliferation potential. Both of experiments indicated SPANXB2 Knockdown impaired PC3 cells proliferation.

Figure 4

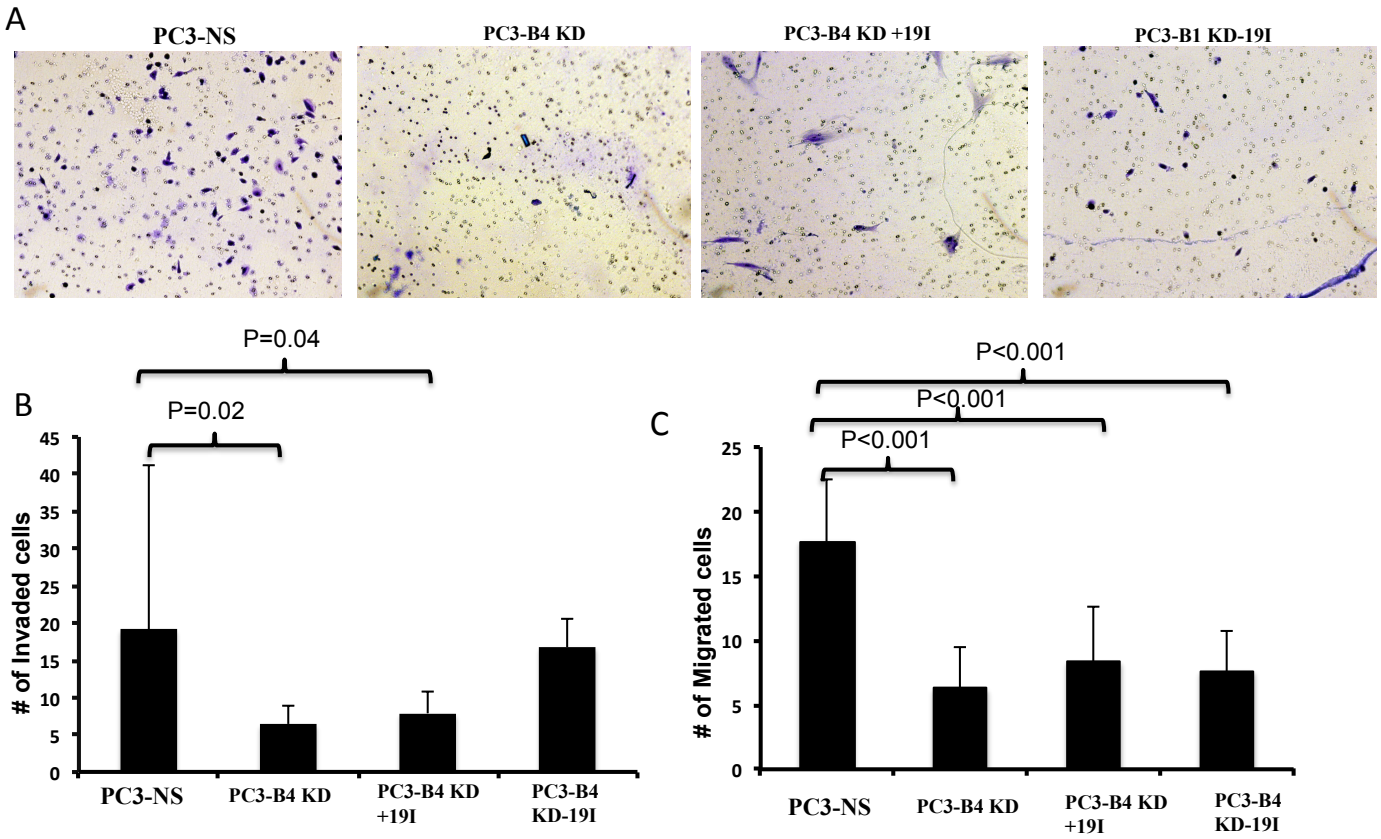

A
PC3-NS
PC3-B1 KD
PC3-B1 KD +19I
PC3-B1 KD-19I
B
Invasion Index
2.5
2
1.5
1
0.5
0
PC3-NS
PC3-B1 KD
PC3-B1 KD +19I
PC3-B1 KD-19I
C
P<0.0001
P<0.0001
P<0.0001
P=0.0156
# of Invaded cells
80
70
60
50
40
30
20
10
0
PC3-NS
PC3-B1 KD
PC3-B1 KD +19I
PC3-B1 KD-19I



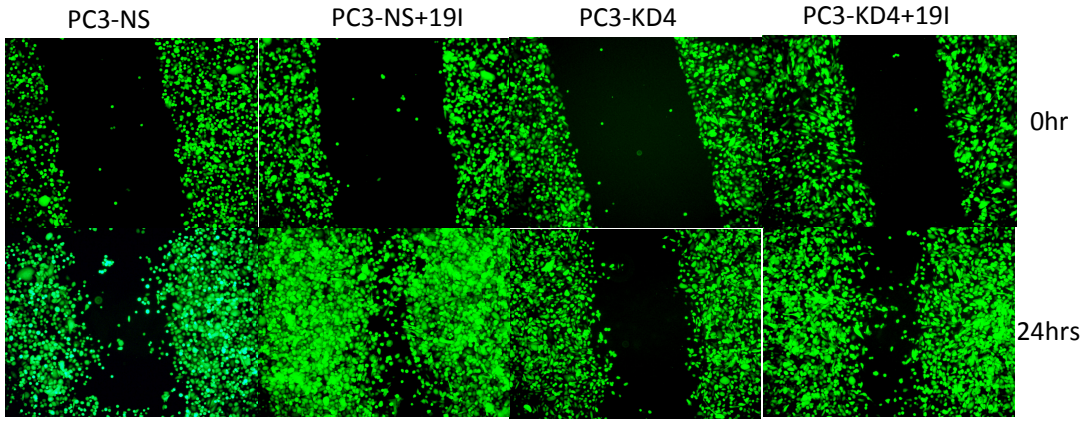
Summary: Figure 4 is linked to the **Task 1a, f&g** : The invasive and migrated potential of SPANXB2 knockdown stale cells were evaluated by BD BioCoat Matrigel Invasion Chambers. PC3-B1, is one of the knockdown clones (see figure 1-3), in which about 58% of SPANXB2 was knockdown compared with non-silence control in mRNA level, was used in this experiment. Four subgroups were assigned: 1) PC3-NS; 2) PC3-B1; 3) PC3 B1 was mixed with HSP 19I (1:1) ; 4) PC3-B1 was recovered from one round PC3-B1 and 19I co-culture model (see my application research part) ; 25,000 cells of each types of cells were put into the inserts except group 3 where 25,000 PC3-B1 and 25,000 19I were mixed and plated into the insert. After 24hours, the invaded cells from the membranes of invasion/migration insert were imaged and counted. A) Represented images of these invasion membranes from each group; B) Invasion index of each cell lines; C) Average invaded cells of each subgroup; statistical bars were presented among these pair that had a significantly difference; D) Migration assay of each subgroup.
<u>These experiments indicated that:</u>

a) <u>In the first time, we illustrated SPANXB2 KD impeded both of invasion and migration ability of PC3 cells.</u>
b) <u>Re-treated/co-cultured these cells with HSP 19I restore or partially restore the cell invasion ability.</u>

Figure 5


A
PC3-NS
PC3-B4 KD
PC3-B4 KD +19I
PC3-B1 KD-19I
B
P=0.04
P=0.02
# of Invaded cells
PC3-NS
PC3-B4 KD
PC3-B4 KD +19I
PC3-B4 KD-19I
C
P<0.001
P<0.001
P<0.001
# of Migrated cells
PC3-NS
PC3-B4 KD
PC3-B4 KD +19I
PC3-B4 KD-19I

D


PC3-NS
PC3-NS+19I
PC3-KD4
PC3-KD4+19I
0hr
24hrs

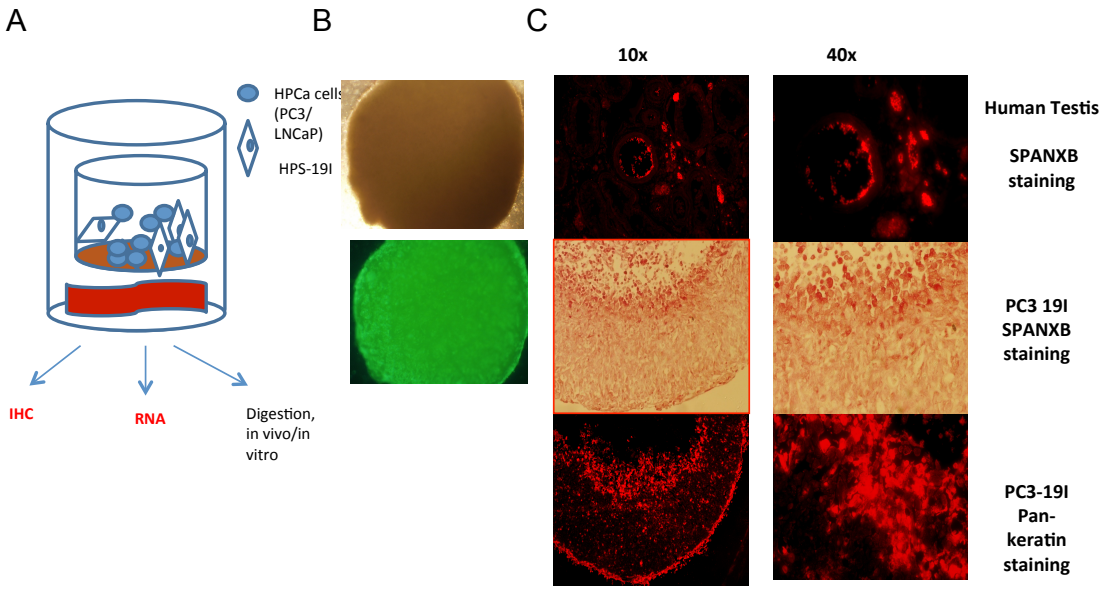
Summary: Figure 5 is linked to the **Task 1a, f&g** : The invasive and migrated potential of SPANXB2 knockdown KD4 ([PC3-SB4) stable cells were evaluated by BD BioCoat Matrigel Invasion Chambers. As same as in figure 4, four subgroups were assigned: 1) PC3-NS; 2) PC3-B4; 3) PC3 B4 was mixed with HSP 19I (1:1) ; 4) PC3-B4 was recovered from one round PC3-B4 and 19I co-culture model (see my application research part) ; 25,000 cells of each types of cells were put into the inserts except group 3 where 25000 PC3-B4 and 25000 19I were mixed and plated into the insert. After 24hours, the invaded cells from the membranes of invasion/migration insert were imaged and counted. A) Represented images of these invasion membranes from each group; B) Average invaded cells of each subgroup; statistical bars were presented among these pair that had a significantly difference; C) Migration assay of each subgroup; D) Scratch assay (wound healing assay) of PC3-NS, PC3-NS-19I (PC3-NS after stromal cells treated), PC3-SB4, PC3-SB4-19I (PC3-SB4 after stromal cells treated),

These experiments indicated that:

a) Further confirmed the influence of SPANXB2 KD on both of invasion and migration abilities of PC3 cells by using another KD clone.
b) Re-treated/co-cultured these cells with HSP 19I restored or partially restore the cell invasion ability.

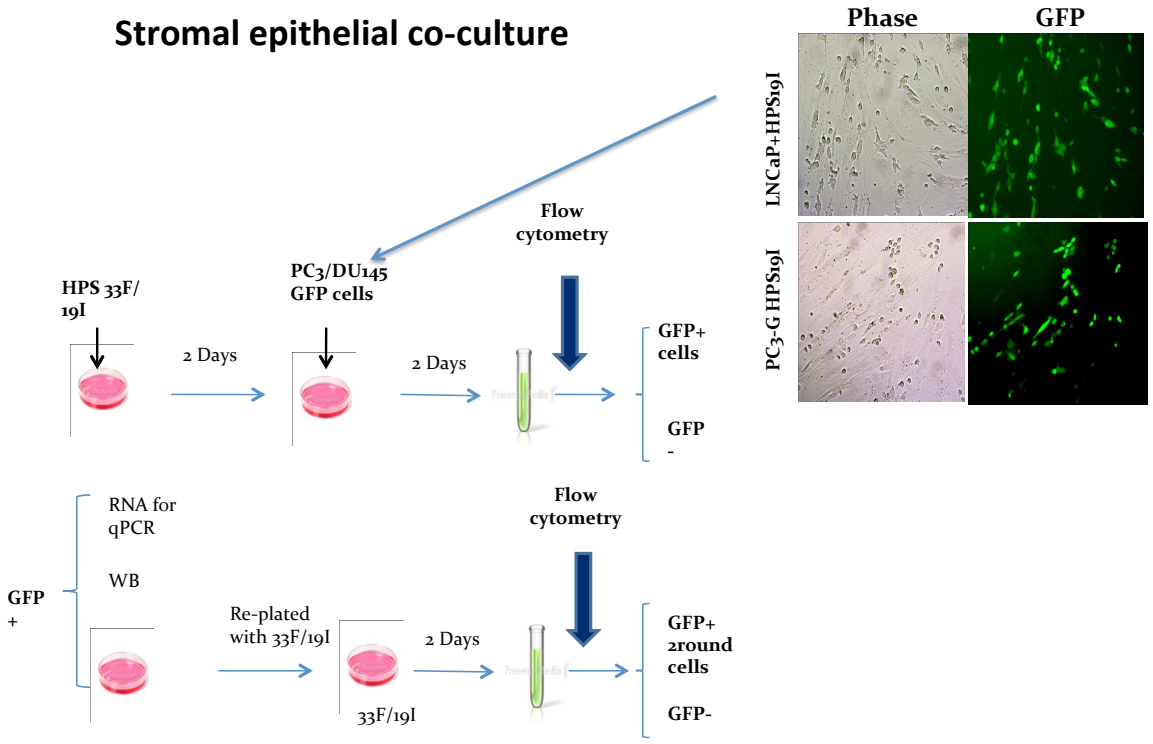
Figure 6

SPANXB expression in Prostate cancer cells /stoma co-culture model II – Organoids



Summary: Figure 6 is linked to the **Task 1B, a, b & c** : A) is the cartoon model of the epithelial-stoma interaction Organoid. Prostate cancer cells are mixed with HSP cells (1:2 ratio) and plated into the top insert, the insert is immersed into the cup which is filled with medium. After 72 hours incubation, a 3-D organoid will be formed. The stromal cells will be inside to form the core, the epithelial cells will be in the out layer. B) Image of an Organoid by PC3-GFP cells with HSP19I. C) Immune staining for SPANXB in a PC3-HSP19I Organoid model, the top is human testis positive control, the middle is the staining of SPANXB in epithelial layer, the bottom image is to confirm the location of epithelia cells by using a Pan-keratin staining. <u>These experiments indicate that SPANXB expression may be enriched in the epithelial layer in a epithelial-stroma 3D model.</u>

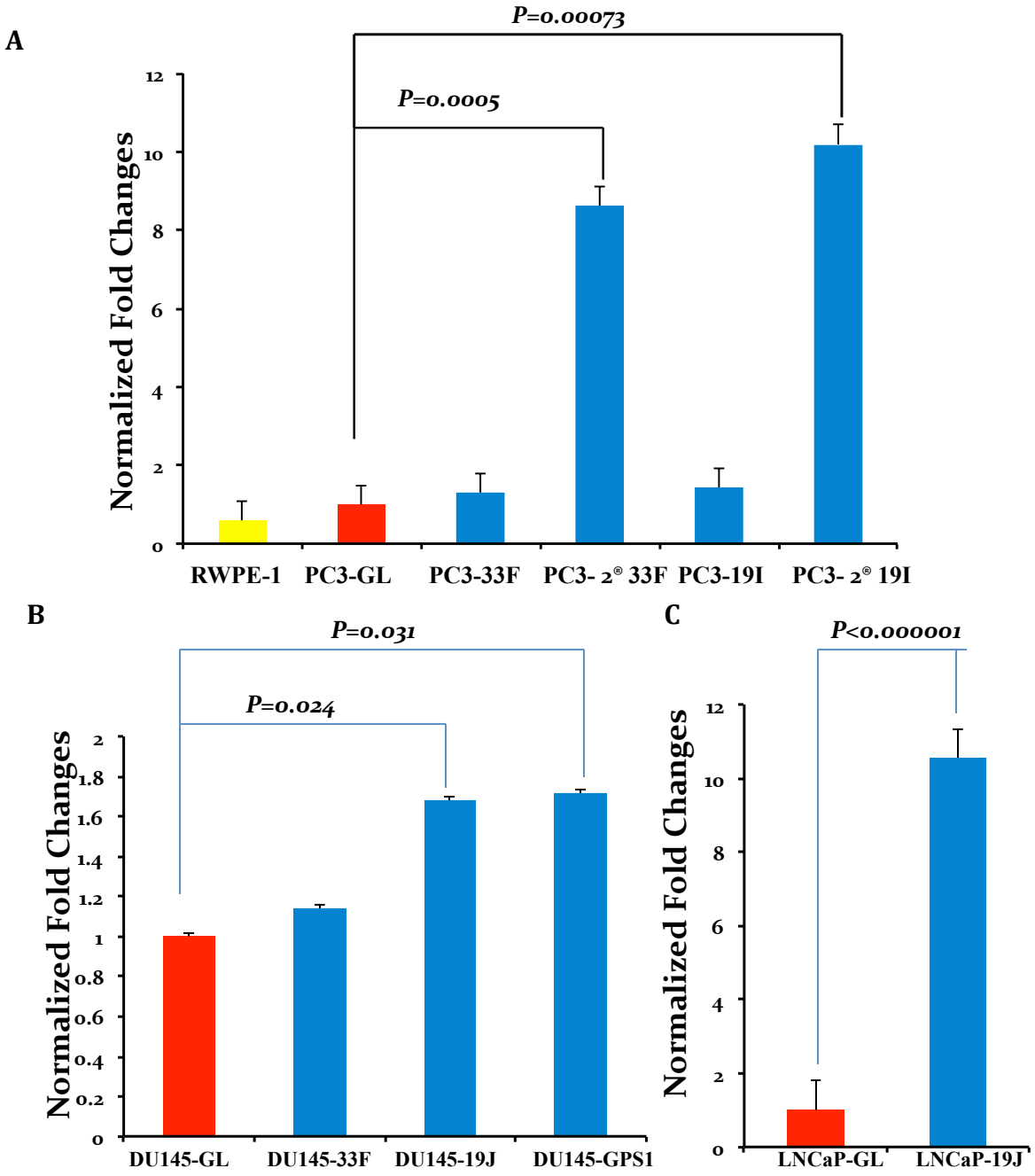
Figure 7



Summary: Figure 7 is linked to the **Task 1B, d & e** : A schematic chart for an *in vitro* stromal-epithelial co-culture model: HPS 19I /33F was pre-cultured for two days, GFP tagged cancer cells then were plated on the top of these cells (1:1 ratio) , the culture medium was switched into cancer cell favorite medium at this moment. After two day's co-culture, the mixture of two type of cells was passed the flow cytometry, both of GFP positive and negative cells were sorted. GFP positive cells will be used for different purpose including isolation of RNA and protein. Some of these cells were continually plated into a stromal cells coated dish, as same as the experiment performed in the first round, then after another two days, these cells will be re-sorted by flow cytometry. The image on the right upper corner indicated the GFP label cancer cells (LNCaP , top ; PC3, lower) were plated on the top of stromal cells.

The advantages of this model lies in following: 1) By comparing with the non-contact epithelia-stroma model (for example, using conditional medium from stromal cells to treat cancer cell) , the direct co-culture model may allow epithelial cells and stromal cells have a fully interaction, the mechanism recovered by this model might be more closed to the real tumor microenvironment; 2) The second round model allows us to further enhance the interaction between epithelial and stroma, it might be critical to observe some important relation between epithelial and stromas which hold a dose/timing-dependent pattern.

Figure 8



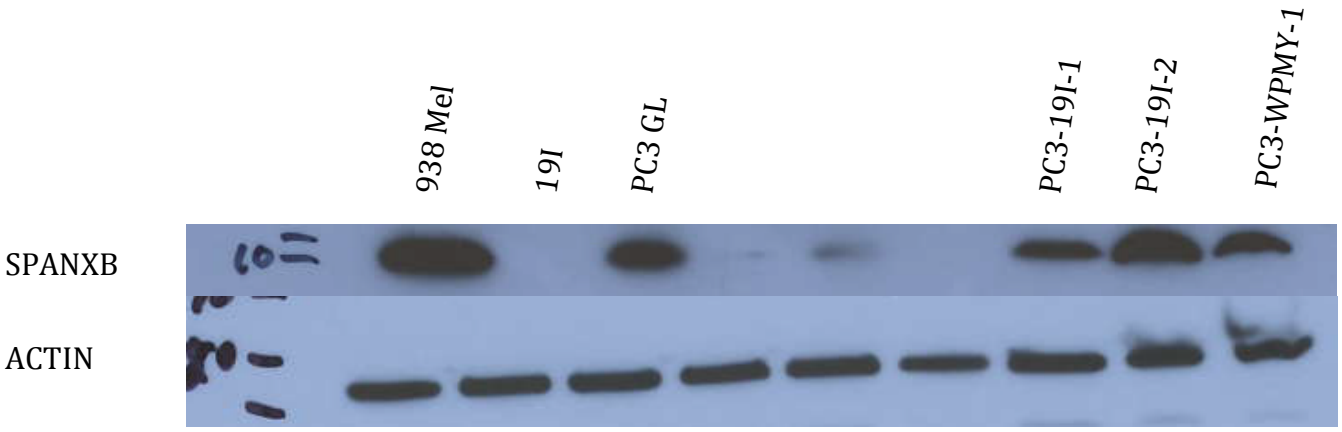
Summary: Figure 8 is linked to the **Task 1B, d**: A: PC3 cells were co-cultured with stromal cells according to the description in Figure 7. GFP positive cells were sorted and RNA was isolated. A real time qPCR was performed to detect the SPANXB2 expression. Unexpectedly, SPANXB2 mRNA level did not significantly increase followed by the first round co-cultured with either HSP 19I or 33F. Surprisingly, the SPANXB2 level significantly raises after the second round co-culture. These strongly imply that enough duration of this co-culture system is required for the activation of SPANXB2 expression in PC3 cells. B) We further test the SPANXB2 expression in other prostate cancer line DU145 and LNCaP (C). Both of Du145 and LNCaP cells

were treated as description in Figure 7, consistently, SPANXB2 level were elevated in both of cells after first round co-culture.

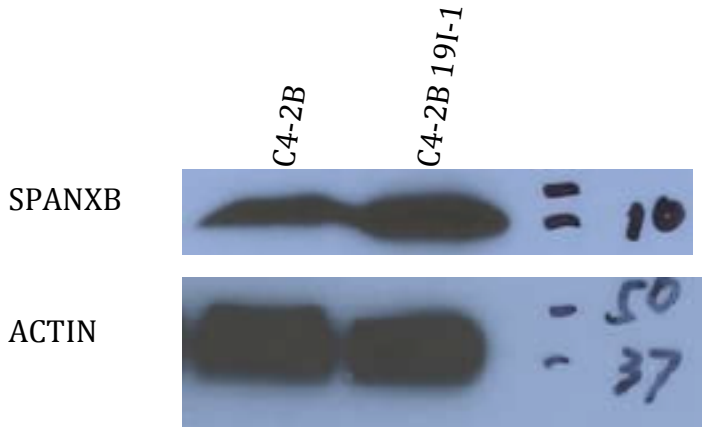
These data in Figure 8 provided strong evidence to support our hypothesis: the epithelial-stroma co-culture activates SPANXB2 expression in prostate cancer lines, while the second round co-culture may be critical for activation of SPANXB2 in PC3 cells.

Figure 9

A



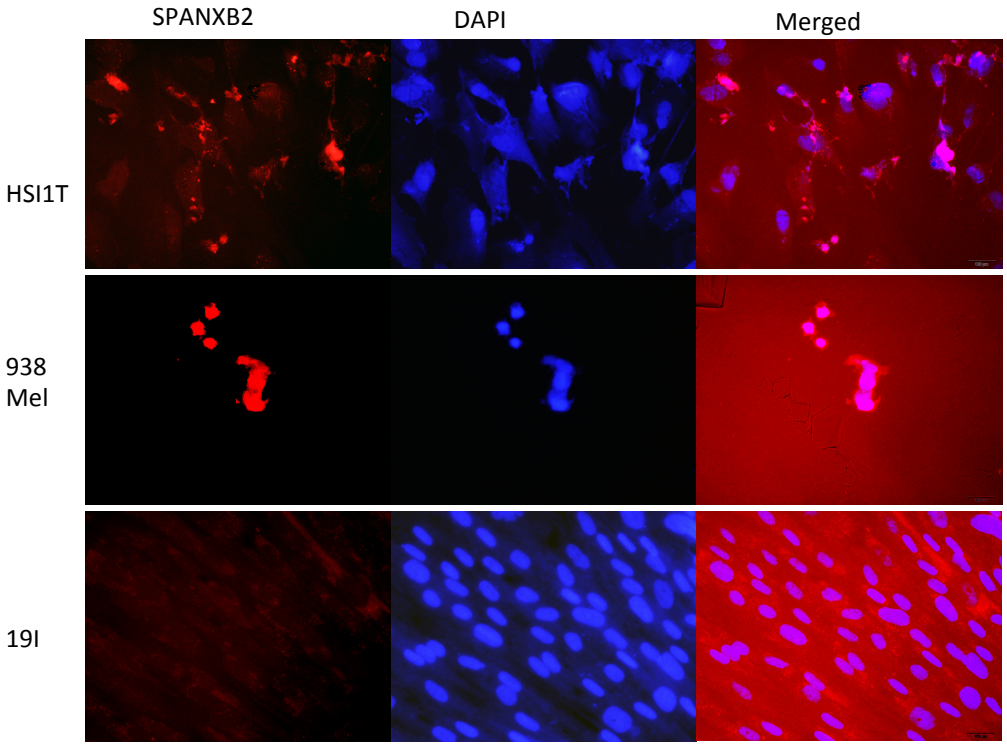
B



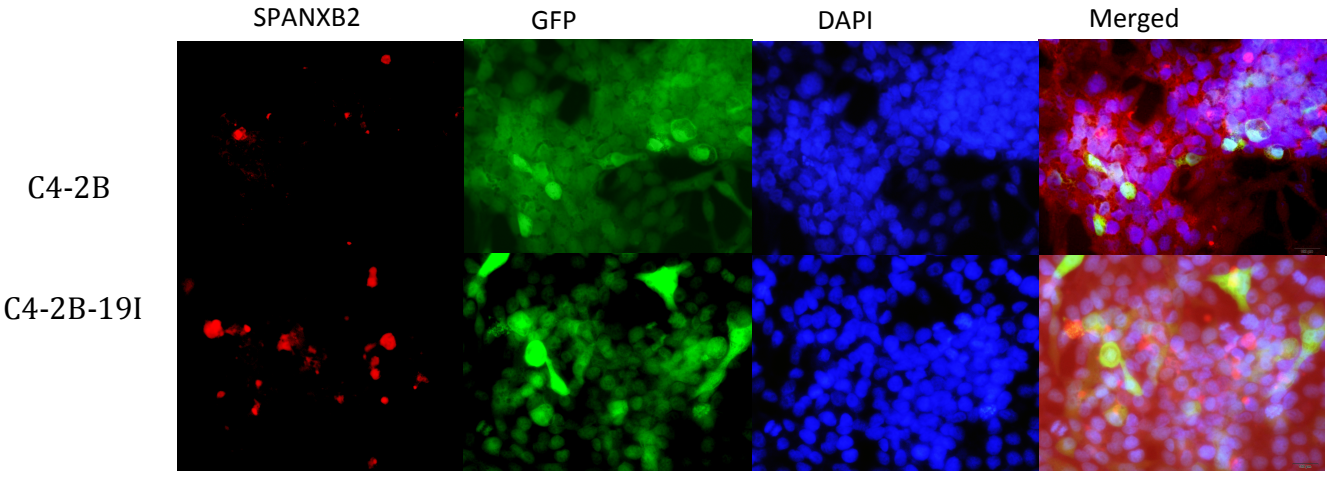
Summary: Figure 9 is linked to the **Task 1B, d**: A: Western blotting for SPANXB were performed in these PC3 cells generated from the co-culture model. PC3-19I-1 is the cell recovered from one round co-culture; PC3-19I-2 is the cells that recovered from *second round* co-culture. PC3-WPMY-1 is cells generated from PC3 and WPMY co-culture model. 938-Mel, a melanoma line, is one of the positive control for SPANXB antibody. The result indicated SPANXB level did not change or increase after the first round co-culture, but it did increased on the second round co-culture. SPANXB protein level is consistent to the qPCR data. Since SPANXB level is none or extremely low, the contamination from 19I stromal cells can be excluded, the increased SPANXB2 level may be solely due to activation in epithelial cells. (B) SPANXB western blotting of C4-2B and C4-2B-19I. It clearly indicated that SPANXB was increased in the co-cultured cells. Both of experiments provide supports in protein level of SPANXB elevation in epithelial-stromal interaction.

Figure 10

A

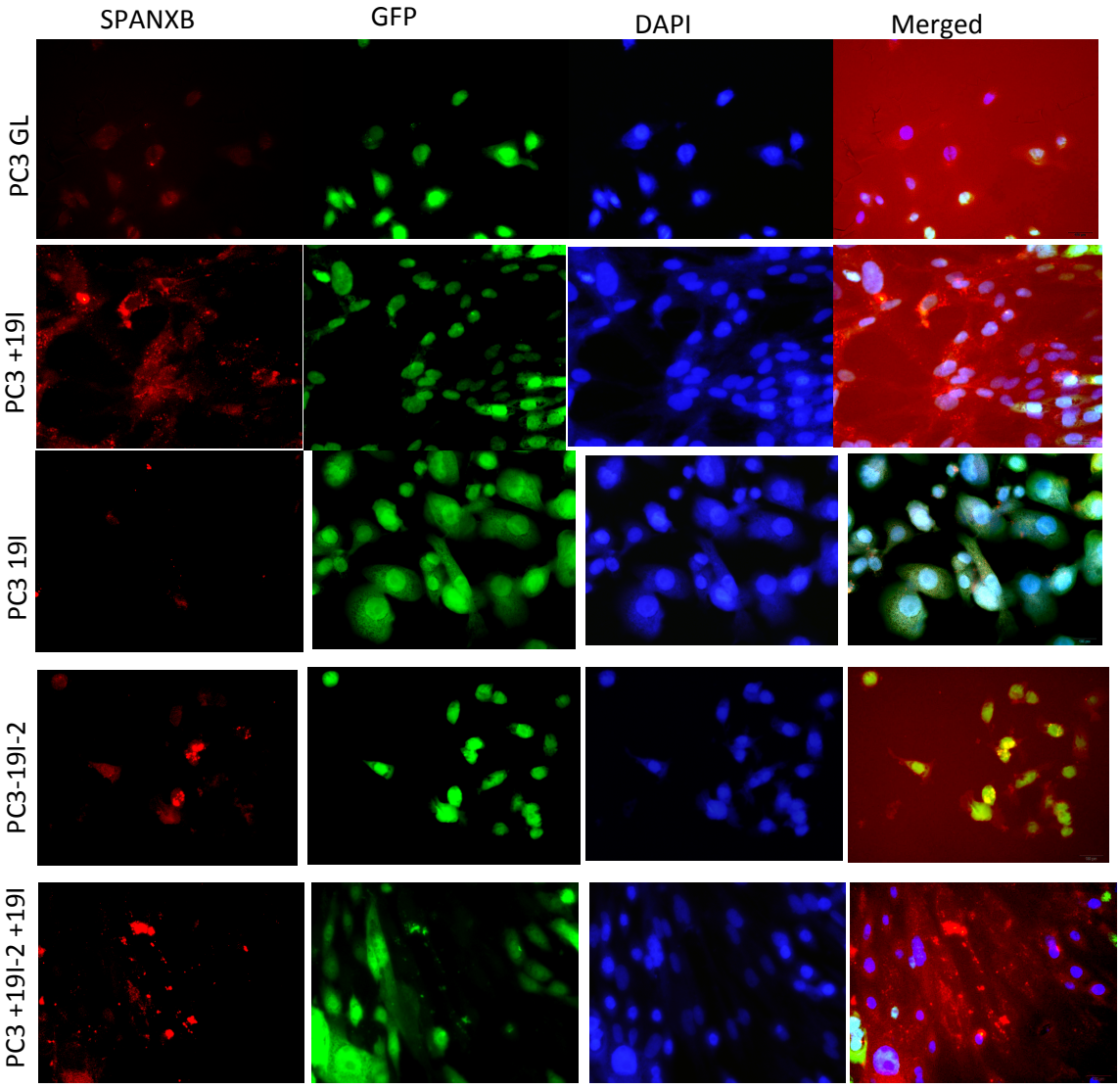


B



Summary: Figure 10 is linked to the **Task 1B, d**: A: Immunofluorescence staining for SPANXB in two positive control line, HSI1T (human testis cell line) and 938-Mel, as well as in 19I. (B) SPANXB IF staining in C4-2B and C4-2B-19I. Immunofluorescence staining result provides further evidence of SPANXB2 elevation in co-culture model.

Figure 11

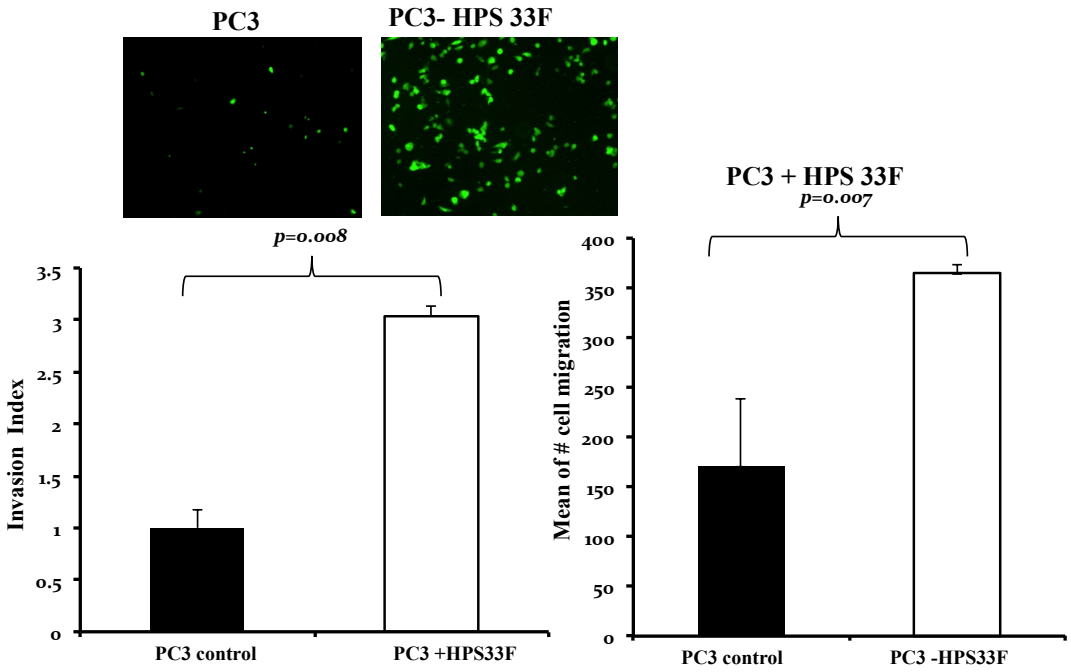


Summary: Figure 11 is linked to the **Task 1B, d**: Immunofluorescence staining for SPANXB in PC3 cells and PC3 co-culture model. From top to lower panel, PC3 GL is the wild type control; PC3 + 19I is the slide that we directly plate PC3 GL cells on the top of 19I cells, it is a mixture of PC3 GL and 19I cells; PC3-19I is the first round co-culture cells, as correlated to the previous qPCR and western blotting data, the staining of SPANXB in PC3-19I is not very strong and there is no obvious enhanced staining by comparing with the wild type control; PC3-19I-2 is the second round co-culture cells and it indicated significantly increase of staining; PC3-19I-2+19I is the co-culture model that we plated PC3-19I-2 on the top of 19I. In this model, we show the largest increase of SPANXB staining. These SPANXB staining in PC3 cells provided consistent results as we previously attained by qPCR and western blotting.

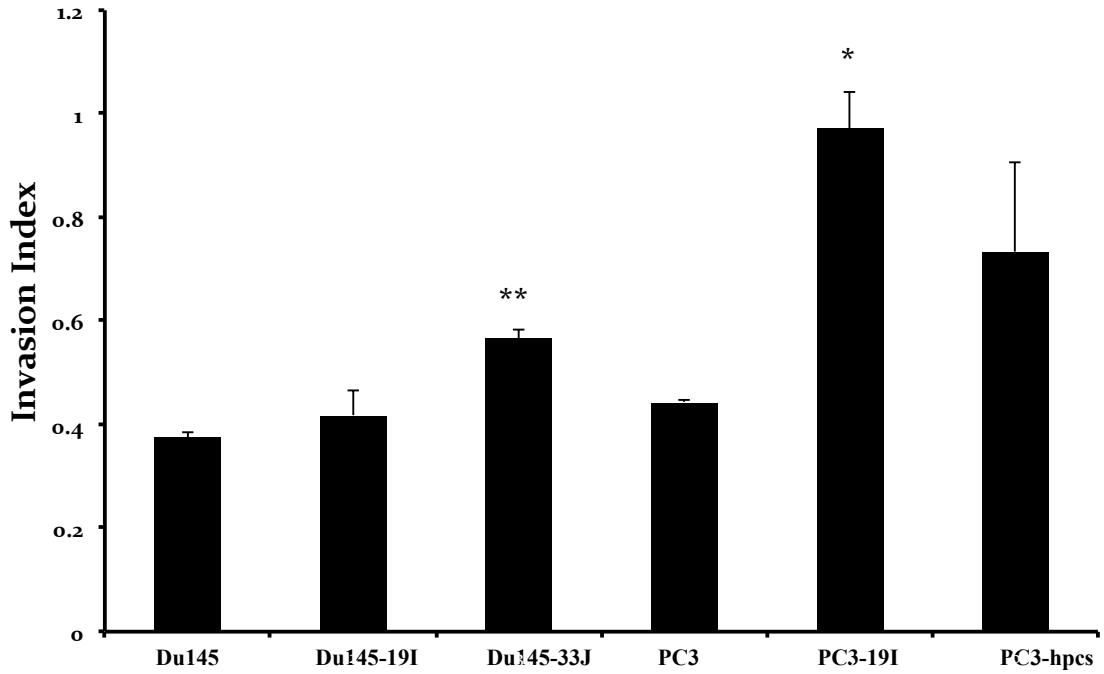
Additionally, due to the SPANXB staining difference between our recovered cells from co-cultured model and these mixed cells of cancer cells and stromal cells, it suggests that the interaction between epithelial and stromal may be complicated, a certain factors, like the incubation time, cells density, may play an important role in this process; On the other hand, it also implied that the directly physical contact might be critical to activate SPANXB2 in cancer cells.

Figure 12

A

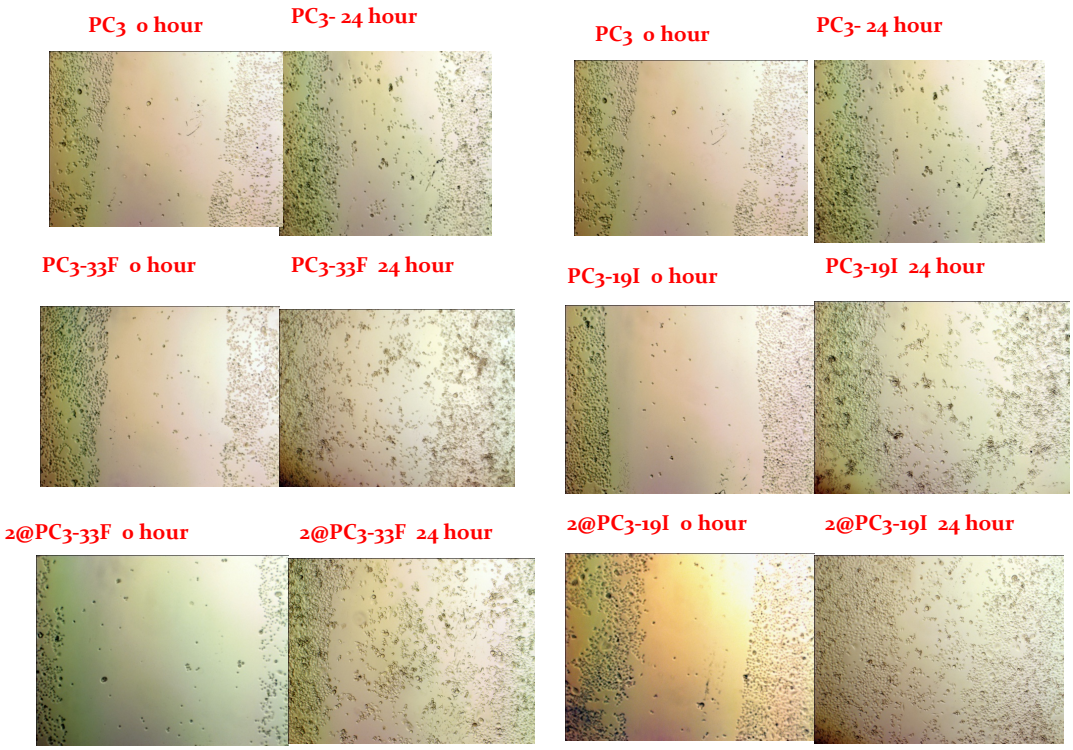

PC3
PC3- HPS 33F
p=0.008
Invasion Index
3.5
3
2.5
2
1.5
1
0.5
0
PC3 control
PC3 +HPS33F
PC3 + HPS 33F
p=0.007
Mean of # cell migration
400
350
300
250
200
150
100
50
0
PC3 control
PC3 -HPS33F

B


Invasion Index
1.2
1
0.8
0.6
0.4
0.2
0
**
*
Du145
Du145-19I
Du145-33J
PC3
PC3-19I
PC3-hpcs

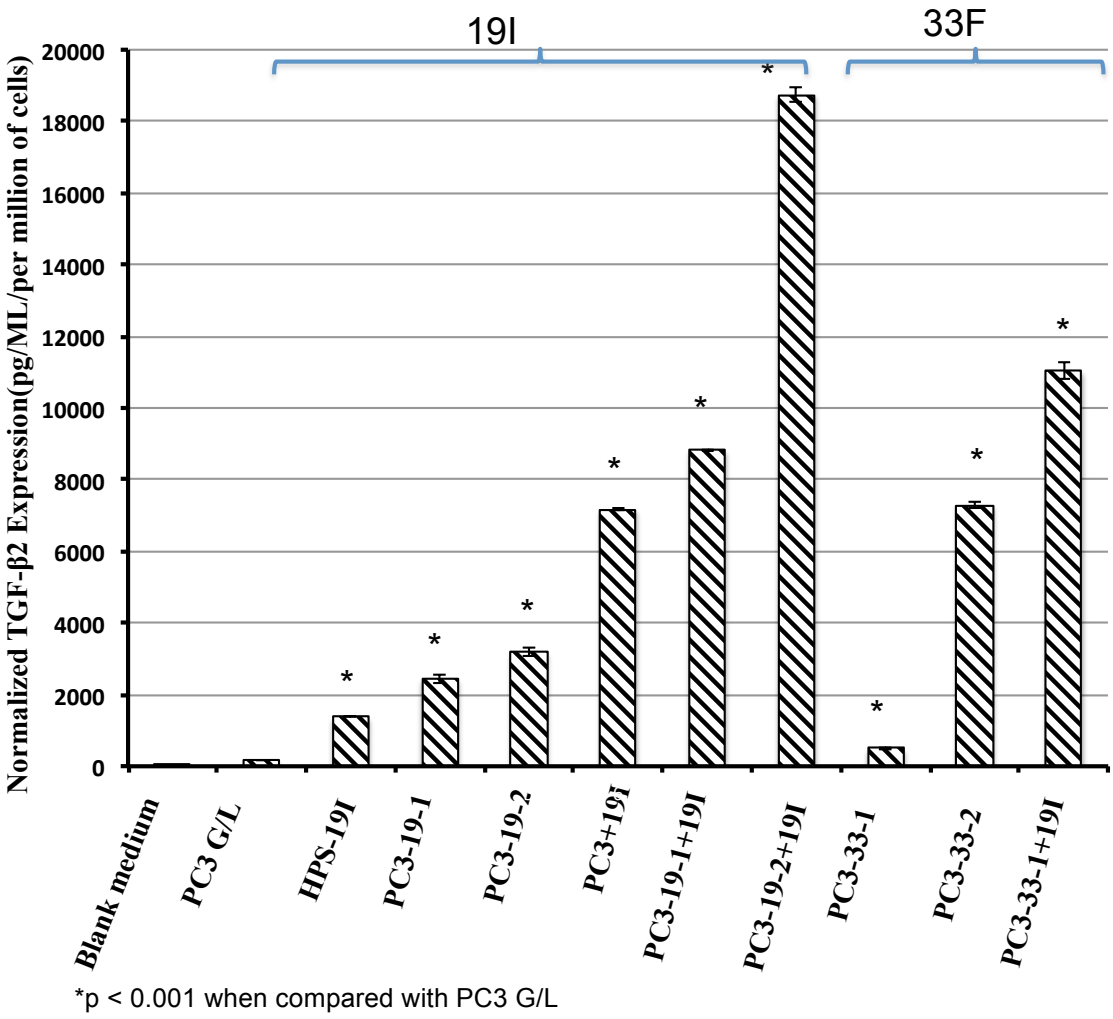
Summary: Figure 12 is linked to the **Task 1B, e**: Invasion assays were performed in these GFP positive cells and their parental cell line as we described in Figure 4. (A) PC3-33F exhibited significant strength in both invasive and migrate ability compared with the PC3 wild type, this is the direct evidence that indicated the epithelial-stromal interaction enhanced the function of malignant cells or aggravated the aggressive behavior of prostate cancer cells, these data are consistent with previous studies (3); these GFP images on the top left show the representative image of invasion membrane from PC3 wild type (left) and PC3-33F (right) respectively; B) Invasion assay were performed in other cancer cell lines co-culture model. A similar result was obtained in PC3-19I model. For DU145 model, DU145-33F exhibited the enhanced invasion while the DU145-19I did not work out. PC3 cells also did not show any increasing of invasion ability by co-culture with a human prostate cancer derived stromal cells. These phenomenon may suggest that these functional changes of cancer cells due to epithelial-stromal interaction be dependent on the type of cancer cells.

Figure 13



Summary: Figure 13 is linked to the **Task 1B, e**: As another proof of this task, a wound healing/scratch assay was also performed. PC3 cells recovered from either first round and second round co-culture model with either 19I or 33F were used in this experiment. In consistent to the invasion assay in Figure 12, PC3-19I-1, PC3-9I-2 demonstrated a gradually increase of migration/wound healing ability, as well as these PC3-33F-1 and PC3-33F-2.

Figure 14



Summary: Figure 14 is linked to the **Task 1B, f,g,h,i**: ELISA assay of TGFβ2 in conditional medium collected from PC3 -19I/33F co-culture model. Two types of model was used in this experiment, one is cancer cells sorted from epithelial-stromal model (see figure 7) , these cells are solely epithelial cells (cancer cells), like PC3-19I-1/2, PC3-33-1 et al; the other one is the mixture of cancer cells and stromal cells, cancer cells did not get separated from stromal cells when analysis was performed, there are still two cell types in these mixture. A "+" was put into the title of these cell line , for example, like PC3+19I , it means PC3 cells was co-cultured with 19I and did not separate each other. Conditional medium was collected from both of these two models.

TGFβ2 ELISA result from this figure strongly support our hypothesis: in our preliminary data for fellowship application, I identified that TGFβ2 but not TGFβ1 induced SPANXB2 expression, we further hypothesized that TGFβ2 may get activation due to epithelial-stromal interaction and therefore stimulated SPANXB2 expression. In this process, TGFβ2 might play important role to modulate SPANXB2

expression. The ELISA data fully supported the hypothesis. In both of two cells type, the isolated sole cancer cells, and the mixture of cancer cell with stromal cells, TGFβ2 level is dramatically increased. It is coincide with other's previous report that TGFβ was activated by interaction between epithelial and stromal cells. This activation exhibited a clearly dose-dependent model, the longer treatment (second round co-culture) , the more elevated TGFβ2 level. More interesting, between the two type of cells, sole cancer cells from co-culture and the mixture of co-culture cells, the later shows a distinctive higher level of TGFβ2 comparing with the former, this result greatly help us understand the potential mechanism of TGFβ2: the activation of TGFβ may largely dependent on the directly (physically) contact between epithelial cells vs stromal cells. Additionally, both of HSP 19I and 33F illustrated the very similar pattern on activation TGF-β2, as well as SPANXB (see previous figure), this suggest that our observation may not be limited only in one stromal cell type, it might be a common mechanism for human prostate stromal cells; Finally, *the most important*, the pattern of TGFβ2 activation is extremely correlated to the pattern of elevated SPANXB in co-culture model (see previous figure 8,9,10,11) . Both the levels of TGFβ2 and SPANXB present very similarly up-regulated with a dose dependent way after co-culture and the directly contact model stimulated the most increased in both of them. This is the so far the strongest evidence to support our hypothesis that TGFβ2 is the underlying mechanism that modulates SPANXB2 expression in this event.

Figure 15

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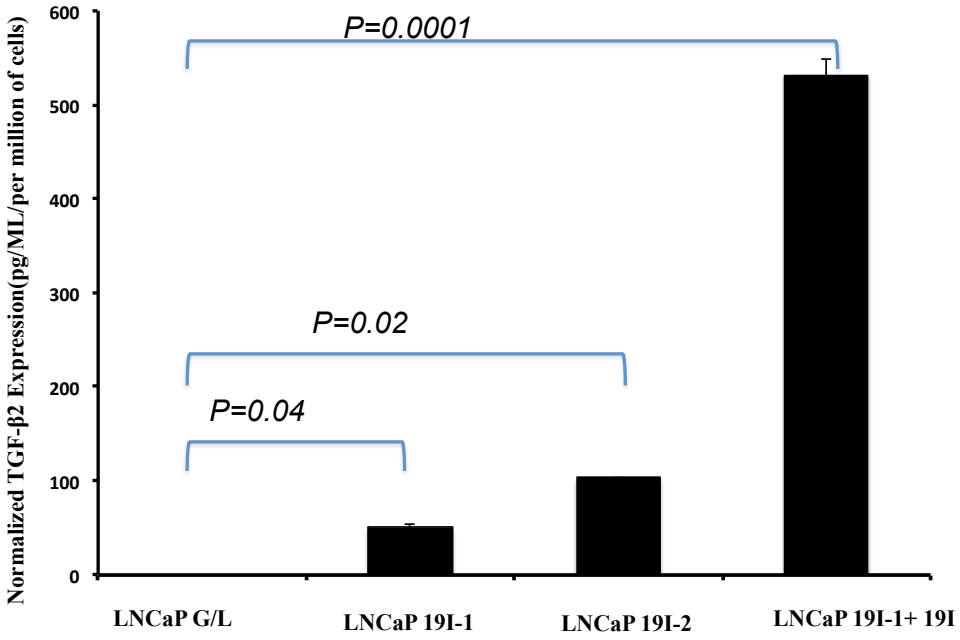

Summary: Figure 15 is linked to the **Task 1B, f,g,h,i**: ELISA assay of TGFβ2 in conditional medium collected from LNCaP-19I co-culture model. TGFβ2 level raise significantly in all of these co-culture cells including LNCaP 19I-1/2 and LNCaP 19I-1 + 19I. The elevation pattern of TGFβ2 in LNCaP-19I co-culture is identical to the pattern we observed in PC3 model. All of these characteristics of PC3 model also present in LNCaP model. Except further confirming our hypothesis, *it also implied that our observation is not only limited to one cancer cell type (PC3) by combing with other evidence we got in our previous figure.*

Key Research Accomplishments

1) Establish PC3-SPANX-B2 knock down stable line (PC3-SPANXB2) and non-sense control line by using shRNA GIPZ lenti-virus vector. (Figure 1)
2) Validate the knockdown effect by using western blotting and qPCR. (Figure 2)
3) Illustrate the knockdown of SPANXB2 suppress ability of proliferation, migration, and invasion in prostate cancer cells. (figure 3-5)
4) Establishment of a serial epithelial-stromas interaction model . (Figure 7)
5) Confirm that the epithelial-stromas interaction increases the SPANXB2 level in cancer cells by qPCR, western blotting and immunofluorescence staining. (Figure 8-11)
6) Demonstrate the stromas promote cancer cells proliferation, migration and invasion. (Figure 12-13)
7) Indicate that TGFβ2 secretion is coincide to the elevated level of SPANXB2. (Figure 14-15)

Reportable Outcomes

Li H, Diallo Krou E., Ressler S., Sreekumar A., Ayala G., Rowley D., Pienta K, Palapattu G. Cancer Testis antigen SPANXB2 and Prostate Cancer Progression. **AUA 2013 Annual Meeting** May 4-8, 2013, San Diego, CA.

Li H, Diallo Krou E., Hernandez J, Ressler S., Sreekumar A., Ayala G., Rowley D., Pienta K, Palapattu G. SPANXB2 expression increases prostate cancer aggressivity: A new view of stromal epithelial cell interaction. **Sixth Annual Prostate Cancer Program Retreat.** March 18-20, 2013, Fort Lauderdale, FL.

Conclusion

1) For the first time, we report that SPANXB2 plays an important role in regulating prostate cancer aggressiveness *in vitro .*
2) Prostate stromas promote SPANXB2 expression, as well as enhance the aggressiveness of cancer cells.
3) TGFβ2 secretion is correlated to the SPANXB2 level, may be the key regulator for this induction of SPANXB2 in epithelial-stromas interaction.
4) All of our *in vitro* studies strongly support us to further confirm these results in our *in vivo* model.

I had successfully accomplished my first year's fellowship work and the results are positive to support my hypothesis.

Reference

1. Almanzar G, Olkhanud PB, Bodogai M, et al. (2009) Sperm-derived SPANX-B is a clinically relevant tumor antigen that is expressed in human tumors and readily recognized by human CD4+ and CD8+ T cells. *Clin Cancer Res*. 2009 Mar 15;15(6):1954-63.
2. Kouprina N, Noskov VN, Pavlicek A, et al (2007) Evolutionary diversification of SPANX-N sperm protein gene structure and expression. *PLoS One.* 2007 Apr 4;2(4)
3. Barron DA, Rowley DR. (2012) The reactive stroma microenvironment and prostate cancer progression. *Endocr Relat Cancer*. Oct 30;19(6):R187-204.

Appendices

None